CRYSTAL STRUCTURE OF ACYL CARRIER PROTEIN SYNTHASE AND ACYL CARRIER PROTEIN SYNTHASE COMPLEX

This application claims the benefit of U.S. Provisional Application No. 60/178,639 filed January 28, 2000.

Background of the Invention

Acyl Carrier Proteins (ACPs) play important roles in a number of biosynthetic pathways that are dependent upon acyl group transfers. They are most often associated with the biosynthesis of fatty acids [1,2], but they are also utilized in the synthesis of polyketide antibiotics [3,4], non-ribosomal peptides [5,6], and of intermediates used in the synthesis of vitamins such as the protein-bound coenzymes, lipoic acid [7] and biotin [8]. The ACP in each of these pathways is composed of 80-100 residues and is either an integrated domain in a larger multi-functional protein (Type I) or is a structurally independent protein that is part of a non-aggregated multi-enzyme system (Type II). Type I ACPs are found in mammals, fungi and certain Mycobacteria while type II ACPs are utilized by plants and most bacteria. The fact that these proteins are essential for the maturation of the organism has led to their investigation as targets for the development of new anti-microbial agents [9-12].

ACPs require post-translational modification for activity. They are converted from an inactive apo-form to an active holo-form by the transfer of the 4'-phosphopantetheinyl (P-pant) moiety of coenzyme A to a conserved serine on the ACP. Evidence now suggests [13] that each ACP that is dependent upon P-pant attachment for activation has its own acyl carrier protein synthase responsible for this attachment.

The post-translational modification of the fatty acid synthase ACP is performed by holo-[acyl carrier protein] synthase (hereinafter defined as "ACPS"; Enzyme Commission No. 2.7.8.7). ACPS produces holo-fatty acid synthase ACP by transferring the P-pant moiety to Ser-36 of the apo-fatty acid synthase ACP in a magnesium dependent reaction [14] as follows:

$$\text{CoA + apo-ACP} \xrightarrow[\text{ACPS, } Mg^{2+}]{} \text{holo-ACP + 3',5'-ADP.}$$

The over-expression and purification of the ACPS from *Escherichia coli* has been described [15] and this protein has been classified as a member of a new enzyme superfamily, the phosphopantetheinyl transferases [13]. Other members of this superfamily have low similarity with *E. coli* ACPS (12-22%), but each has been shown to possess P-pant transferase activity. Alignment of these proteins show that two regions, residues 5-13 and 51-65 (*E. coli* ACPS numbering), are highly conserved with eight of the residues in these regions being strictly conserved.

While numerous members of the phosphopantetheinyl transferase superfamily have been identified and sequenced, until the present invention, no one, to the inventors' knowledge, has discovered the crystal structure of an ACPS-like phosphopantetheinyl transferase or has characterized the three dimensional structure of the molecule's Co-A active site. Determination of the three dimensional structure of ACPS and its CoA active site is critical to the rational identification and/or design of therapeutic or antibiotic agents that may act as inhibitors or activators of ACPS enzymatic activity. Alternatively, using conventional drug assay techniques, the only way to identify such an agent is to screen thousands of test compounds, either in culture or by administration to suitable animal models in a laboratory setting, until an agent having the desired inhibitory or activating effect on a target compound is identified. Necessarily, such conventional screening methods are expensive, time consuming, and do not elucidate the method of action of the identified agent on the target compound.

However, advancing X-ray, spectroscopic and computer modeling technologies allow researchers to visualize the three dimensional structure of a targeted compound. Using such a three dimensional structure, researchers identify putative binding sites and then identify or design agents to interact with these

binding sites. These agents are then screened for an activating or inhibitory effect upon the target molecule. In this manner, not only are the number of agents to be screened for the desired activity greatly reduced, but the mechanism of action on the target compound is better understood. Further, the three dimensional structure of one compound determined using these techniques can be used to ascertain the three dimensional structure of other related compounds.

Recently, Reuter, *et al.* have disclosed the crystal structure of another member of the P-pant transferase superfamily, Sfp, complexed with CoA [22]. Sfp converts the inactive apo forms of the seven PCP domains of surfactin synthetase to their active holo-forms by transfer of the 4'-phosphopantetheinyl moiety of CoA to the side chain hydroxyl of a serine residue found in PCP domains. Thus, Sfp is essential in the production of lipoheptapeptide antibiotic surfactin in *B. subtilis* [22].

The "Sfp-like" P-pant transferases are very different than the "ACPS-like" P-pant transferases. In particular, the Sfp-like P-pant transferases activate PCP (peptidyl carrier protein) domains of various non-ribosomal peptide synthetases and are present in monomeric form. Further, Sfp shows a pseudo 2-fold symmetry dividing the molecule into two similarly folded halves of roughly identical size. In contrast, the ACPS-like P-pant transferases, which form homodimers, activate the ACP domains or subunits of fatty acid synthetases, polyketide synthases and other enzymes, and are about half the size of Sfp-like transferases. While the pseudo 2-fold symmetry of the Sfp synthetase activating enzyme disclosed by Reuter, et al. suggests that dimerization may be necessary for the formation of an intact ACPS-like P-pant transferase [22], the crystal structure of Sfp, alone or complexed with CoA, is not sufficient to generate a three dimensional model of an ACPS-like P-pant transferase nor is it useful for designing or identifying agents which may activate or inhibit ACPS enzymatic activity. Furthermore, as discussed below, there are significant differences between the Sfp

and ACPS structures that clearly place the two enzymes in different functional groups of the P-pant transferase superfamily.

Summary of the Invention

The present invention relates to a crystallized ACPS-like phosphopantetheinyl (P-pant) transferase, and in particular, to an acyl carrier protein synthase (ACPS), as well as to a crystallized complex comprising acyl carrier protein synthase and coenzyme A (CoA) (hereinafter referred to as "ACPS-CoA complex"). The invention is further directed to the three dimensional structure of the ACPS-like P-pant transferases, including ACPS and the ACPS-CoA complex, as determined using crystallographic analysis (with or without sedimentation analysis) of ACPS and the ACPS-CoA complex. Particularly, the invention is directed to the three dimensional structure of the CoA binding site present in ACPS and other ACPS-like P-pant transferases that mediates P-pant attachment from CoA to various carrier proteins, alone and as complexed with CoA or other agents that interact with the CoA binding site of said transferases. Identification of the three dimensional structure of the CoA binding site will be valuable for the design of antibiotics and other agents that interfere with P-pant attachment, thereby preventing activation of corresponding carrier proteins.

The invention additionally provides a method for identifying an agent that interacts with any active site of ACPS, comprising the steps of determining a putative active site of ACPS from a three dimensional model of the ACPS enzyme, and by performing various computer fitting analyses to identify an agent which interacts with the putative active site. Such agents may act as inhibitors or activators of ACPS activity, as determined by obtaining the identified agent, contacting the same with ACPS and measuring the agent's effect on ACPS activity. Similarly, the present invention also provides a method for identifying an agent that interacts with any active site of an ACPS-CoA complex, comprising the steps of

determining a putative active site of an ACPS-CoA complex from a three dimensional model of the ACPS-CoA complex, and performing various computer fitting analyses to identify an agent which interacts with the putative active site. Again, such agents may act as inhibitors or activators of ACPS-CoA complex activity, as determined by obtaining the identified agent, contacting the same with ACPS-CoA complex, and measuring the agent's effect on ACPS-CoA complex activity.

Yet another aspect of the present invention is a method for identifying an activator or inhibitor of any molecule or molecular complex which comprises a CoA binding site, including any member of the ACPS-like P-pant transferases, comprising the steps of generating a three dimensional model of said molecule or molecular complex using the relative structural coordinates according to Figure 1 or 2 of residues ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and of ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, ± a root mean square deviation from the backbone atoms of said residues of not more than 1.5Å, and then selecting or designing a candidate activator or inhibitor that interacts with said molecule or molecular complex using computer fitting analyses of interactions between the three dimensional model of the molecule or molecular complex and the candidate activator or inhibitor. The effect of the candidate activator or inhibitor may be evaluated by obtaining the candidate activator or inhibitor, contacting the same with the molecule or molecular complex, and measuring the effect of the candidate activator or inhibitor on molecular or molecular complex activity.

Alternatively, the three dimensional model of the molecule or molecular complex comprising a CoA binding site may be determined using the relative structural coordinates according to Figure 1 or 2 of residues ARG53,

ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and ASP8, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, or alternatively, of residues LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å. Also provided by the present invention are the activators or inhibitors selected or designed using the above-noted methods.

Still further, the present invention is directed to a method of determining the three dimensional structure of a molecule or molecular complex whose structure is unknown, comprising the steps of first obtaining crystals of the molecule or molecular complex whose structure is unknown, and then generating X-ray diffraction data from the crystallized molecule or molecular complex. The X-ray diffraction data from the molecule or molecular complex is compared with the known three dimensional structures determined from the ACPS and/or ACPS-CoA crystals of the present invention, and molecular replacement analysis is used to conform the known three dimensional structures to the X-ray diffraction data from the crystallized molecule or molecular complex.

Finally, the present invention provides the CoA active site of an ACPS-like P-pant transferase, including, but not limited to, an ACPS, comprising, alternatively, (a) the relative structural coordinates according to Figure 1 of ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean

square deviation from the backbone atoms of said amino acids of not more than 1.5Å, (b) the structural coordinates according to Figure 1 of residues ARG53, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and ASP8, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or (c) the structural coordinates according to Figure 1 of residues LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å.

In an additional embodiment, the present invention provides the CoA active site of an ACPS-like P-pant transferase, including, but not limited to, an ACPS, wherein said active site is in its bound configuration, and comprising alternatively, (a) the relative structural coordinates according to Figure 2 of ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, (b) the structural coordinates according to Figure 2 of residues ARG53, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and ASP8, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not

more than 1.5Å, or (c) the structural coordinates according to Figure 2 of residues LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å.

Brief Description of the Figures

Figure 1 lists the atomic structure coordinates for ACPS as derived by X-ray diffraction of an ACPS crystal. "Atom type" refers to the atom whose coordinates are being measured. "Residue" refers to the type of residue of which each measured atom is a part - i.e., amino acid, cofactor, ligand or solvent. The "x, y and z" coordinates indicate the Cartesian coordinates of each measured atom's location in the unit cell (Å). "Occ" indicates the occupancy factor. "B" indicates the "B-value", which is a measure of how mobile the atom is in the atomic structure ($Å^2$). "MOL" indicates the segment identification used to uniquely identify each molecule.

Figure 2 lists the atomic structure coordinates for ACPS and CoA as derived by X-ray diffraction of an ACPS-CoA crystal. Figure headings are as noted above.

Figure 3A is a diagram showing the arrangement of the six monomers in the monoclinic ACPS structure into two trimers in the asymmetric unit. Figure 3B is the stereo image of the ribbon diagram of the ACPS molecule from the ACPS-CoA complex structure, with the secondary structure elements labeled according to the Richardson diagram shown in Figure 3C.

Figure 4 is a stereo diagram showing how the β-sheets from the ACPS molecule interact to form a three-faced β-sandwich in the trimer.

Figure 5 illustrates interactions among the coenzyme A molecule, the ACPS protein and solvent molecules.

Figures 6A and 6B illustrate results from equilibrium ultracentrifugation experiments using 100 μM and 100 μM ACPS/200 μM CoA, at pH 6.4 and 25°C.

Figure 7 depicts a non-reducing, denaturing gel of ACPS samples before and after analytical centrifugation experiments.

Figure 8 depicts the amino acid sequence of ACPS isolated from *B. subtilis*, deposited as SWISS-PROT accession number P96618.

Figure 9 illustrates the alignment of amino acid sequences for twelve members of the ACPS family, including the consensus sequence.

Detailed Description of the Invention

As used herein, the following terms and phrases shall have the meanings set forth below:

"ACPS" includes acyl carrier protein synthases as well as "ACPS-like" P-pant transferases. Acyl carrier protein synthases produce a holo-fatty acid synthase ACP by transferring the P-pant moiety to Ser-36 (or equivalent Serine) of an apo-fatty acid synthase ACP in a magnesium dependent reaction. "ACPS-like" P-pant transferases are those enzymes having P-pant transferase activity (i.e., that transfer the 4'-phosphopantetheinyl moiety of CoA to a conserved serine on the corresponding target molecule) which form homodimers and activate the ACP domains or subunits of fatty acid synthases, polyketide synthases or other enzymes.

Unless otherwise indicated, "protein" shall include a protein, polypeptide or peptide.

An "agent" shall include a protein, polypeptide, peptide, nucleic acid, including DNA or RNA, molecule, compound, antibiotic or drug.

"Root mean square deviation" is the square root of the arithmetic mean of the squares of the deviations from the mean, and is a way of expressing deviation or variation from the structural coordinates of ACPS and ACPS-CoA described herein.

It will be obvious to the skilled practitioner that the numbering of the amino acid residues in the various isoforms of ACPS or in other ACPS-like P-pant transferases may be different than that set forth herein. Corresponding amino acids and conservative substitutions in other isoforms or P-pant transferases are easily identified by visual inspection of the relevant amino acid sequences or by using commercially available homology software programs. "Conservative substitutions" are those amino acid substitutions which are functionally equivalent to the substituted amino acid residue, either by way of having similar polarity, steric arrangement, or by belonging to the same class as the substituted residue (e.g., hydrophobic, acidic or basic).

The present invention is directed to a crystallized ACPS-like P-pant transferase, and more specifically, to a crystallized acyl carrier protein synthase enzyme, that effectively diffracts X-rays for the determination of the structural coordinates of the enzyme. The invention further provides a crystallized ACPS-CoA complex that effectively diffracts X-rays for the determination of the structural coordinates of the ACPS-CoA complex.

As used herein, the protein used in the ACPS crystals and crystal complexes of the present invention includes any protein (i.e., as used herein, any protein, polypeptide or peptide), isolated from any source (including, but not limited to, a protein isolated from *Aquifex*, *Chlamydophila*, *Helicobacter*, *Staphylococcus*, *Thermotoga*, *Escherichia*, *Rickettsia*, *Streptomyces*, *Treponema*, *Bacillus*, *Bradyrhizobium*, and *Mycobacterium*), wherein said protein has ACPS-like

P-pant transferase activity, and further comprises the consensus sequence as shown in Figure 9. Additionally, the protein used in the ACPS crystals and crystal complexes of the present invention includes proteins having ACPS-like P-pant transferase activity which comprise the relative structural coordinates according to Figure 1 or 2 for the residues GLY6, ASP8, ALA51, LYS57, GLU58, ARG53, ALA59, LYS62 and ALA63, ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å. In a preferred embodiment of the invention and as exemplified below, ACPS is cloned and isolated from *B. subtilis*, and then overexpressed in a commercially available *E. coli* system.

In an alternate embodiment of the present invention, the ACPS used to generate the crystals and/or crystal complexes of the present invention comprises amino acid residues ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, ARG45, PHE49, ARG53, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68, PHE74, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105, or conservative substitutions thereof. These amino acids constitute a depression which defines the CoA active site in the three dimensional structure of the ACPS enzyme, wherein the depression is more particularly comprised of the relative structural coordinates according to Figure 1 or 2 of residues ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and residues ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second molecule of ACPS, ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å.

There are six ACPS molecules in the asymmetric unit of the ACPS crystal. In one embodiment of the invention, the relative structural coordinates according to Figure 1 of the two monomers forming the depression defining the

CoA active site are of residues ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from ACPS1, and residues ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from ACPS2. In alternate embodiments, the relative structural coordinates according to Figure 1 are from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

In an alternate preferred embodiment, the ACPS used to generate the crystals and/or crystal complexes of the present invention comprises amino acid residues which are within 4Å of the CoA molecule associated with the ACPS CoA binding site. In a specific embodiment, the ACPS comprises amino acid residues ASP8, PHE25, ARG28, ILE29, ARG53, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68, PHE74, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105, or conservative substitutions thereof. Such residues specifically comprise the relative structural coordinates according to Figure 1 or 2 of residues ARG53, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and ASP8, PHE25, ARG28, ILE29, PHE24, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68, and PHE74 from a second monomer of ACPS, ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å. In alternate embodiments, the relative structural coordinates according to Figure 1 are from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

In yet another alternate preferred embodiment, the ACPS used to generate the crystals and/or crystal complexes of the present invention comprises

amino acid residues which are within 4Å to 8Å of the CoA molecule associated with the ACPS CoA binding site. Specifically, such residues include ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71, LEU72, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111, or conservative substitutions thereof. Such residues more particularly comprise the relative structural coordinates according to Figure 1 or 2 of residues LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å (or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å), and more specifically may comprise the relative structural coordinates of residues according to Figure 1 from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

Further, the ACPS used to generate the crystals and/or crystal complexes of the present invention may comprise the entire 121 amino acid residues of Figure 8, and the structural coordinates of these residues according to Figure 1 or 2, ± a root mean square deviation from the backbone atoms of said amino acid residues of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å.

The crystals of the present invention may take a wide variety of forms, all of which are included in the present invention. However, in a preferred

embodiment of the present invention, the crystallized ACPS enzyme is characterized as being in plate form with space group $P2_1$, and having unit cell parameters of a=76.26Å, b=76.16Å, c=85.69Å, and beta=93.3°, and further consists of six molecules of ACPS in the asymmetric unit. Similarly, in a preferred embodiment of the present invention, the crystallized ACPS-CoA complex is characterized as being in pyramidal form with space group R3, and having unit cell parameters of a=b=55.82Å and c=92.28Å, and further consists of one molecule of ACPS and one molecule of CoA in the asymmetric unit.

Once a crystal or crystal complex of the present invention is grown, X-ray diffraction data can be collected by a variety of means in order to obtain the atomic coordinates of the crystallized molecule or molecular complex. With the aid of specifically designed computer software, such crystallographic data can be used to generate a three dimensional structure of the molecule or molecular complex. Various methods used to generate and refine the three dimensional structure of a crystallized molecule or molecular structure are well known to those skilled in the art, and include, without limitation, multiwavelength anomalous dispersion (MAD), multiple isomorphous replacement, reciprocal space solvent flattening, molecular replacement, and single isomorphous replacement with anomalous scattering (SIRAS).

The three dimensional structure of the preferred ACPS enzyme of the present invention exists in its active state as a trimer, wherein each copy of the ACPS molecule comprises amino acid residues 1-118 as shown in Figure 8. The core of the enzyme is an α helix (α4) composed of residues 43 to 64, which runs the entire length of the protein. One side of this helix is covered by an antiparallel β sheet (the A-sheet), with topology β1, β5 and β4. A β ribbon composed of strands β3 and β2, combine with α3 to cover another side of helix α4. The encasement of α4 is completed by α1, α2 and a loop consisting of residues 66-75.

Molecular modeling methods known in the art may be used to identify an active site of the ACPS molecule or ACPS molecular complex. The identification of putative active sites of a molecule or molecular complex is of great importance, as most often the biological activity of a molecule or molecular complex results from the interaction between an agent and one or more active sites of the molecule or molecular complex. Accordingly, the active sites of a molecule or molecular complex are the best targets to use in the design or selection of activators or inhibitors that affect the activity of the molecule or molecular complex.

As used herein, an "active site" refers to a region of a molecule or molecular complex that, as a result of its shape and charge potential, interacts with another agent (including, without limitation, a protein, polypeptide, peptide, nucleic acid, including DNA or RNA, molecule, compound, antibiotic or drug). The agent may be an activator or inhibitor of the molecular or molecular complex activity. The present invention is directed to a CoA active site of an ACPS-like P-pant transferase, including the active site of an acyl carrier protein synthase, comprising the relative structural coordinates according to Figure 1 of residues ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and of ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å. More specifically, the active site of ACPS in its native (i.e., unbound) state may comprise the relative structural coordinates of the residues according to Figure 1 from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

In an alternate embodiment, the CoA active site of the present invention comprises the relative structural coordinates according to Figure 1 of residues ARG53, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and of residues ASP8, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å. The active site may comprise the relative structural coordinates of the residues according to Figure 1 from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

In a yet further embodiment, the CoA active site of the present invention comprises the relative structural coordinates according to Figure 1 of residues LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and of residues ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably, not more than 1.0Å, and most preferably, not more than 0.5Å. The active site may comprise the relative structural coordinates of the residues according to Figure 1 from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

Further still, an alternate embodiment of the CoA active site of the present invention comprises the relative structural coordinates according to Figure

1 of GLY6, ASP8, ALA51, ARG53, LYS57, GLU58, ALA59, LYS62 and ALA63, ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably, not more than 1.0Å, and most preferably, not more than 0.5Å.

The invention is further directed to a CoA active site of an ACPS-like P-pant transferase, including an acyl carrier protein synthase, in its "bound state", i.e., configured in a state of association or interaction with an agent, wherein the agent may be a protein, polypeptide, peptide, nucleic acid, including DNA or RNA, molecule, compound, antibiotic or drug. In one preferred embodiment of the invention, the active site of the present invention is configured in a state of association or interaction with CoA.

In a preferred embodiment of the invention, the CoA active site in its bound state comprises the relative structural coordinates according to Figure 2 of residues ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and of ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å.

In an alternate embodiment, the active site comprises the relative structural coordinates according to Figure 2 of residues ARG53, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and of residues ASP8, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å.

In a yet further embodiment, the active site comprises the relative structural coordinates according to Figure 2 of residues LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and of residues ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably, not more than 1.0Å, and most preferably, not more than 0.5Å.

Finally, a CoA active site of the present invention comprises the relative structural coordinates according to Figure 2 of GLY6, ASP8, ALA51, ARG53, LYS57, GLU58, ALA59, LYS62 and ALA63, ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably, not more than 1.0Å, and most preferably, not more than 0.5Å.

Another aspect of the present invention is directed to a method for identifying an agent that interacts with an active site of ACPS, including a CoA active site in either its native or bound state, comprising the steps of determining an active site of ACPS from a three dimensional model of the ACPS enzyme and performing computer fitting analyses to identify an agent which interacts with said active site. Computer fitting analyses utilize various computer software programs that evaluate the "fit" between the putative active site and the identified agent, by (a) generating a three dimensional model of the putative active site of a molecule or molecular complex using homology modeling or the atomic structural coordinates of the active site, and (b) determining the degree of association between the putative active site and the identified agent. Three dimensional models of the putative active site may be generated using any one of a number of methods known in the art, and include, but are not limited to, homology modeling as well as computer analysis of raw data generated using crystallographic or

spectroscopy data. Computer programs used to generate such three dimensional models and/or perform the necessary fitting analyses include, but are not limited to: GRID (Oxford University, Oxford, UK), MCSS (Molecular Simulations, San Diego, CA), AUTODOCK (Scripps Research Institute, La Jolla, CA), DOCK (University of California, San Francisco, CA), Flo99 (Thistlesoft, Morris Township, NJ), Ludi (Molecular Simulations, San Diego, CA), QUANTA (Molecular Simulations, San Diego, CA), Insight (Molecular Simulations, San Diego, CA), SYBYL (TRIPOS, Inc., St. Louis. MO) and LEAPFROG (TRIPOS, Inc., St. Louis, MO).

The effect of such an agent identified by computer fitting analyses on ACPS activity may be further evaluated by contacting the identified agent with ACPS and measuring the effect of the agent on ACPS activity. Depending upon the action of the agent on the active site of ACPS, the agent may act either as an inhibitor or activator of ACPS activity. Standard enzymatic assays may be performed and the results analyzed to determine whether the agent is an inhibitor of ACPS activity (i.e., the agent may reduce or prevent binding affinity between ACPS and the relevant substrate, such as ACP or CoA, and thereby reduce the level or rate of ACPS activity compared to baseline), or an activator of ACPS activity (i.e., the agent may increase binding affinity between ACPS and the relevant substrate molecule, such as ACP or CoA, and thereby increase the level or rate of ACPS activity compared to baseline). Further tests may be performed to evaluate the effect of the identified agent on bacterial or eukaryotic cell populations, wherein an inhibitor of ACPS activity inhibits cell viability or reproduction.

A similar method may be used in order to identify an agent that interacts with an active site of ACPS-CoA complex, including an ACP active site, comprising the steps of determining an active site of the ACPS-CoA complex from a three dimensional model of the ACPS-CoA complex, and performing computer fitting analyses to identify an agent which interacts with said active site. Again, the

effect of the agent on ACPS-CoA complex activity may be further evaluated by contacting the identified agent with ACPS-CoA complex and measuring the effect of the agent on ACPS-CoA complex activity using standard enzymatic assays. Depending upon the action of the agent on the active site of the ACPS-CoA complex, the agent may act either as an inhibitor (by reducing or preventing binding affinity between ACPS-CoA and the relevant substrate, such as ACP, and thereby reducing the level or rate of ACPS-CoA activity compared to baseline) or activator (by increasing or enhancing binding affinity between ACPS-CoA and the relevant substrate, such as ACP, and thereby increasing the level or rate of ACPS-CoA activity compared to baseline). Further tests may be performed to evaluate the effect of the identified agent on bacterial or eukaryotic cell populations, wherein an inhibitor of ACPS-CoA activity inhibits cell viability or reproduction.

The present invention is not limited to identifying agents which interact with an active site of ACPS or ACPS-CoA complex, but also is directed to a method for identifying an activator or inhibitor of any molecule or molecular complex comprising a CoA binding site, comprising the first step of generating a three dimensional model of said molecule or molecular complex comprising a CoA binding site using the relative structural coordinates according to Figure 1 or 2 of residues ARG45, PHE49, ARG53, LYS81, ASN84, GLY85, LYS86, PRO87, ILE103, THR104 and HIS105 from one monomer of ACPS, and of ASP8, GLU11, ARG14, MET18, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å. In alternate embodiments, the relative structural coordinates according to Figure 1 are from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4

and ACPS6, respectively. Then, a candidate activator or inhibitor is selected or designed by performing computer fitting analyses of said candidate agent with the three dimensional model of the molecule or molecular complex comprising a CoA active site. Once the candidate activator or inhibitor is obtained, it may be contacted with the molecule or molecular complex in order to measure the effect the candidate activator or inhibitor has on said molecule or molecular complex.

Alternatively, the three dimensional structure of the molecule or molecular complex comprising a CoA binding site may be determined using (a) the relative structural coordinates according to Figure 1 or 2 of residues ARG53, ASN84, GLY85, LYS86, PRO87, ILE103, THR104, and HIS105 from one monomer of ACPS and of residues ASP8, PHE25, ARG28, ILE29, PHE54, GLU58, SER61, LYS62, GLY65, THR66, GLY67, ILE68 and PHE74 from a second monomer of ACPS, or (b) of LEU41, ARG45, GLU48, PHE49, LEU50, ALA51, GLY52, ILE79, ARG80, LYS81, ASP82, GLN83, TYR88, VAL101, SER102, THR106, TYR109, ALA110, and ALA111 from one monomer of ACPS and of residues ILE5, GLY6, LEU7, ILE9, THR10, ARG14, ILE15, MET18, GLN22, ALA55, LYS57, ALA59, PHE60, ALA63, PHE64, GLY69, ARG70, GLN71 and LEU72 from a second monomer of ACPS, in each case ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5Å, or more preferably not more than 1.0Å, or most preferably, not more than 0.5Å. Again, in alternate embodiments, the relative structural coordinates according to Figure 1 are from ACPS1 and ACPS2, respectively; from ACPS2 and ACPS3, respectively; from ACPS1 and ACPS3, respectively; from ACPS4 and ACPS5, respectively; from ACPS5 and ACPS6, respectively; and from ACPS4 and ACPS6, respectively.

The present invention is also directed to the activators or inhibitors identified using the foregoing methods.

Finally, the present invention is further directed to a method for determining the three dimensional structure of a molecule or molecular complex

whose structure is unknown, comprising the steps of obtaining crystals of the molecule or molecular complex whose structure is unknown and generating X-ray diffraction data from the crystallized molecule or molecular complex. The X-ray diffraction data from the molecule or molecular complex is then compared with the known three dimensional structure determined from the ACPS and/or ACPS-CoA crystals of the present invention. Then, the known three dimensional structure determined from the crystals of the present invention is "conformed" using molecular replacement analysis to the X-ray diffraction data from the crystallized molecule or molecular complex. Alternatively, spectroscopic data or homology modeling may be used to generate a putative three dimensional structure for the molecule or molecular complex, and the putative structure is refined by conformation to the known three dimensional structure determined from the ACPS and/or ACPS-CoA crystals of the present invention.

The present invention may be better understood by reference to the following non-limiting Examples. The following Examples are presented in order to more fully illustrate the preferred embodiments of the invention, and should in no way be construed as limiting the scope of the present invention.

Example 1

(i) Material and Methods

Expression and Purification of ACPS and SeMet-ACPS: ACPS, cloned into pBAD/HIS, was expressed in DH10B *Escherichia coli* at 37°C. Cells were grown in a Biostat C-10 (10L) vessel (B. Braun Biotech) using rich media and induced four hours with 0.2% final arabinose. SeMet labeled expression of ACPS.pBAD/His was carried out in LeMaster media in BL21DE3 *Escherichia coli* at 37°C. These cultures were also induced for 4 hours with 0.2% final arabinose at log phase.

20 grams of wet *E. coli* BL21(DE3)/pML-7 cells expressing ACPS were resuspended in 200 ml basic buffer A (50 mM Hepes·Cl, pH 7.4, 250 mM NaCl,

10 mM $MgCl_2$, 10 mM DTT) and lysed by two passages through a Microfluidizer (Microfluidics Corporation, Newton, MA). Cellular debris was removed by centrifugation at 15,000 g for 30 min. The soluble extract was then loaded onto a 2x20-cm Poros PI (PerSeptive Biosystems, Framingham, MA) column pre-equilibrated with basic buffer A. The flow-through material was applied onto a 2x20-cm Poros HS (PerSeptive Biosystems, Framingham, MA) column pre-equilibrated with the same buffer. The column was washed with a large amount of basic buffer A until no protein was present in the flow-through as judged by Bradford protein assay. ACPS was then eluted with a linear 0.25 -1.0 M NaCl gradient. ACPS containing fractions were pooled and dialyzed against acidic buffer A (50 mM NaAc, pH 4.6, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT) overnight, and applied onto a 0.46 x 10 cm Poros HS column pre-equilibrated with the same acidic buffer A. Greater than 95% pure ACPS was eluted with a linear 0.05-2.0 M NaCl gradient. The fractions were subjected to SDS-PAGE analysis and good fractions were combined and concentrated before loading onto an TSK G2000 (TosoHaas, Montgomeryville, PA) equilibrated with buffer containing 50 mM NaAc, pH 4.6, 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM DTT. ACPS containing fractions were concentrated and used for crystallization. SeMet-ACPS was purified using the same procedure.

Crystallization of ACPS: Prior to crystallization, the protein was dialyzed against a solution containing 10 mM Sodium Acetate pH 4.4, 2 mM $MgCl_2$, 100 mM NaCl, 5 mM DTT and then concentrated to ~10 mg/mL according to the Bradford method [17]. Crystallization conditions for ACPS were determined using the sparse matrix screens available from both Hampton Research and Emerald Biostructures. All screening was done using hanging-drop vapor diffusion by mixing 1 μL well plus 1 mL protein at both 18°C and 4°C. Diffraction quality plate-like crystals were obtained from 2.5 M NaCl, 0.1 M Tris pH 7.0, 0.2 M $MgCl_2$.

These crystals belonged to space group $P2_1$ with cell dimensions of a=76.26Å, b=76.16Å, c=85.69Å, beta=93.3° and contained six molecules of ACPS in the asymmetric unit. The SeMet ACPS also crystallized using these conditions with the exception that the concentration of DTT was increased to 10 mM to help protect against oxidation of the Selenium atoms.

Crystallization of ACPS with Coenzyme A: The protein solution was prepared as above but 24 hours prior to setting up the screening trays, 1 mM Coenzyme A (di-sodium salt, Fluka) was added. The sparse matrix screens from Hampton Research and Emerald Biostructures were again utilized at 18 and 4°C with 2 μL hanging drops. Diffraction quality CoA-ACPS co-crystals were obtained from 0.2 M $CaCl_2$ and 20% PEG. These crystals belonged to space group R3 with cell dimensions a=b=55.82Å, c=92.28Å and contained one molecule of ACPS and one molecule of CoA in the asymmetric unit. The selenomethionine ACPS-CoA crystallized under the same conditions with the exception that the concentration of DTT was increased to 10 mM to help protect against oxidation of the Selenium atoms.

Native Data Collection: Single-wavelength (1.2Å) data for the native protein crystals were collected on beamline 5.O.2 at the ALS, Berkley. A single crystal, cooled to -180°C, was used for each data set. In preparation for cryo-cooling, the $P2_1$ form of ACPS was slowly equilibrated to 30% glycerol. Crystals of the ACPS-CoA complex did not tolerate slow equilibration so they were dipped quickly through synthetic mother liquor, which had the PEG concentration increased to 30%, and then cooled quickly to -180°C. Using the ADSC Quantum-4 CCD detector, the data to 1.4Å were collected for the ACPS-CoA complex crystal and data to 1.8Å were collected for the ACPS crystal using one-degree oscillations. The data were processed using DENZO and Scalepack [18] and the statistics from refinement are given in Table 1.

MAD Data Collection: MAD data were collected on beamline X12-C at the NSLS (Brookhaven National Laboratory, Upton, NY) from a single crystal of SeMet-ACPS, $P2_1$ form and from a single crystal of the SeMet-ACPS/CoA complex, R3 form. Each crystal was cooled to -180°C for data collection as described above. Prior to each data collection the wavelength necessary for the experiment was determined by examining an X-ray fluorescence spectrum in the vicinity of the K-absorbance edge of the Selenium atom. Diffraction data were then collected at the inflection point of the spectrum, the peak and at a wavelength remote from the peak for each crystal. The wavelengths used can be found in Table 1. The data to 1.9Å were collected for the ACPS-CoA complex crystal and the data to 2.4Å were collected for the ACPS crystal using one degree oscillations with the single-module Brandeis CCD detector. These data were then used as input to the program SOLVE [19] for local scaling of the data sets and determination of the Selenium atom positions. SOLVE was able to determine the position of each of the six Selenium atoms in the asymmetric unit of the ACPS crystal and the single site for the Selenium in the ACPS-CoA crystal. Heavy atom parameters for each were refined with SHARP [20].

Model Building and Refinement: The structure of the ACPS monoclinic form was built into the original 2.5Å resolution solvent flattened symmetry-averaged MAD map using the X-AUTOFIT features within QUANTA (Molecular Simulations, Inc.). For symmetry averaging, a section of the protein, which corresponded to the 3-member beta sheet, was rotated and translated onto the density corresponding to the other five protein molecules in the asymmetric unit. The NCS operators were then determined using program LSQKAB and refined further with DM (both programs from the CCP4 suite). The resulting map was of sufficiently high quality that only residues 1, 18-25, and 118-121 were not fit initially. The phases were then extended from 2.5Å to 2.2Å in 1000 cycles using a separate run of DM.

Investigation of the resolution-extended electron density maps for monomers 2 through 6 showed that residues 18-25 were now traceable and that their positions differed between the structures. Alternate tracings of residues 80-99 were also visible between monomers.

This model was then used as the initial model for refinement using the program CNS [21] against the native data, which extended to 1.8Å, rather than the Se-Met MAD data. Following seven cycles of refining and rebuilding, the refinement converged with a model which contained 6 molecules of ACPS and 505 solvent molecules at a Rcryst of 19.6% and a Rfree of 21.9%. The refinement statistics are given in Table 1.

The structure of Monomer 1 of the monoclinic ACPS structure was placed into the solvent averaged MAD map calculated from the ACPS-CoA data and rebuilt. During this rebuilding, extra electron density was observed in the vicinity of HIS105. This density was compared with the structure of CoA and it was quite clear that it did indeed correspond to CoA. The initial model, which did not contain the CoA, was refined using CNS against the 1.5Å native data. After two cycles of rebuilding and refining, the CoA was placed into density. Refinement converged after four additional rebuilding cycles with a R_{cryst} of 18.5% and a R_{free} of 20.1%. The final model consisted of residues 1-118, CoA, 99 waters, two Ca^{2+} and one Cl^-. The refinement statistics are given in Table 1.

Table 1. Statistics for Data Collection, Phase Determination, and Refinement

Data Collection								
	ACPS MAD			**ACPS-CoA MAD**			**ACPS**	**ACPS-CoA**
	Remote	*Peak*	*Inflection*	*Remote*	*Peak*	*Inflection*	*Native*	*Native*
Wavelength(Å)	λ3=0.93	λ1=0.97853	λ2=0.97860	λ3=0.93	λ1=0.97836	λ2=0.97858	λ=1.2	λ=1.2
resolution range(Å)	15-2.4	15-2.5	15-2.4	15-1.9	15-1.9	15-1.9	20-1.8	100-1.5
R_{merge}[a]	8.0%(25.8)	6.8%(15.5)	7.8%(29.4)	6.0%(36.1)	5.9%(21.4)	5.7%(20.5)	7.6%(46.8)	6.4%(30.7)
% complete	100(100)	100(100)	100(100)	99.9(100)	99.9(100)	99.6(100)	96.4(68.6)	99.9(98.9)
total reflections	432961	390215	432248	121119	118498	110614	614385	121535
unique reflections	38299	33941	38335	8174	6982	7031	87902	17170
I/σ(I)	26.1(7.03)	31.9(12.7)	28.4(8.1)	33.2(13.1)	36.9(16.9)	35.8(16.7)	15.1(1.9)	18.1(4.0)
f'(e^-)[b]	-2.18	-7.35	-9.5	-2.18	-7.35	-9.52		
f''(e^-)	3.46	5.92	3.15	3.46	5.92	3.15		

MAD Phasing	**ACPS**								
Resolution Limits(Å)	6.46	4.8	3.99	3.49	3.13	2.87	2.67	2.5	Overall
Phasing Power[c]									
λ3 anomalous	2.88	2.01	1.39	1.03	0.8	0.59	0.46	0.35	0.93
λ1 isomorphous	0.003	0.003	0.003	0.003	0.003	1.86	1.48	1.2	0.003
λ1 anomalous	3.85	3.10	2.09	1.59	1.29	1.01	0.80	0.64	1.45
λ2 isomorphous	6.96	5.15	3.34	2.54	2.03	1.64	1.25	0.03	2.19
λ2 anomalous	2.37	1.81	1.28	1.00	0.77	0.60	0.46	0.01	0.80
Mean FOM[d]	0.77	0.71	0.58	0.48	0.39	0.29	0.21	0.15	0.38

MAD Phasing	**ACPS-CoA**								
Resolution Limits(Å)	6.73	4.41	3.50	2.99	2.64	2.40	2.21	2.06	Overall
Phasing Power									
λ3 anomalous	3.50	2.50	1.99	1.83	1.42	1.14	0.83	0.66	1.42
λ1 isomorphous	1.57	1.22	1.11	1.16	1.12	1.11	1.01	0.92	1.10
λ1 anomalous	4.54	3.42	2.87	2.64	2.11	1.61	1.22	0.97	2.04
λ2 isomorphous	1.53	1.00	0.67	0.53	0.45	0.36	0.37	0.24	0.51
λ2 anomalous	2.81	2.29	1.98	1.70	1.40	1.14	0.85	0.68	1.37
Mean FOM	0.62	0.51	0.51	0.50	0.44	0.38	0.28	0.12	0.38

Table 1. cont'd

Model Refinement	ACPS	ACPS-CoA
Maximum Resolution(Å)	1.8	1.5
R_{work}[c] (%)	19.6	18.6
R_{free} (%)	21.9	20.1
<B value> ($Å^3$)	30.6	17.5
R.m.s. Deviations from ideal geometry for		
Bonds(Å)	0.007	0.008
Angles(°)	1.34	1.31
B values ($Å^2$)	2.185	2.189
Non-hydrogen Protein Atoms	6972	1147
Water Molecules	471	99
Ions	8 sodium, 19 chlorine	2 Calcium
Other Molecules	5 DTT, 2 Glycerol	1 CoA

[a] $\mathbf{R_{merge}} = \sum |\mathbf{I_h} - \langle \mathbf{I_h} \rangle| / \mathbf{I_h}$, where $\langle \mathbf{I_h} \rangle$ is the average intensity over symmetry equivalents. Number in parentheses reflect statistics for the last shell

[b] f' and f" reported values were refined by SHARP.

[c] Phasing power = $\sum |\mathbf{F_H}| / \sum ||\mathbf{F_{PH\,obs}}| - |\mathbf{F_{PHcalc}}||$, where $\mathbf{F_H}$ is the calculated heavy atom structure factor amplitude.

[d] Figure of merit = $\langle \mathbf{P}(\alpha)\mathbf{e}^{i\alpha} / \mathbf{P}(\alpha) \rangle$, where α is the phase and $\mathbf{P}(\alpha)$ is the phase probability distribution.

[e] $\mathbf{R_{work}} = \sum ||\mathbf{F_{obs}}| - |\mathbf{F_{calc}}| / |\mathbf{F_{obs}}|$, $\mathbf{R_{free}}$ is equivalent to $\mathbf{R_{work}}$, but calculated for a randomly chosen 5% (or 10%) of reflections omitted from the refinement process.

(ii) Results

Structure Determination: Large plate-like crystals (0.5 x 0.5 x 0.15 mm) of ACPS were grown in hanging drops using 2.5 M NaCl, 0.2 M $MgCl_2$, 0.1 M Tris pH 7.0. These crystals belong to space group $P2_1$ with unit cell parameters a=76.26Å, b=76.16Å, c=85.69Å, beta=93.3° and diffract to 2.2Å in-house using a R-Axis IV mounted on a Rigaku RUH2R rotating anode operating at 5kW or beyond 1.8Å using synchrotron radiation. Assuming a molecular weight of 14.8 kDa, a protein density of 1.34 g/mL, and six molecules in the asymmetric unit, the Matthew's coefficient is 2.8 $Å^3$/Dalton for a solvent content of 55.7%.

The selenomethionine ACPS protein was expressed in *E. coli* so that the structure could be solved using multi-wavelength anomalous dispersion (MAD) phasing [16]. The selenomethionine ACPS crystallized under identical conditions as the wild-type protein. Data at three different wavelengths around the Se K edge (see Table 1) were collected from a single crystal of the Se-Met ACPS, $P2_1$ form on beamline X12-C of the National Synchrotron Light Source at Brookhaven Laboratory. Experimental phases were determined at 2.5Å, modified by solvent flattening, non-crystallographic symmetry averaging and extended to 2.2Å. This resulted in an experimental map that was of exceptionally high quality.

When the protein is incubated with 1 mM CoA di-sodium salt for 24 hours prior to crystallization, crystals are no longer obtained using the conditions optimized for the ACPS alone. Instead, screening found that this material crystallized from 0.2 M $CaCl_2$ and 20% PEG3350. Similar to the crystals obtained without CoA these crystals showed strong diffraction in-house (to 1.9Å) and using synchrotron radiation, diffraction was seen beyond 1.4Å. These crystals are small pyramids (0.1 x 0.1 x 0.1 mm) which grow in space group R3 with cell dimensions a=b=55.82Å and c=92.28Å. Once again, assuming a molecular weight of 14.8 kDa, a protein density of 1.34 g/mL, and one molecule in the asymmetric unit, the Matthew's coefficient is 1.87 $Å^3$/Dalton for a solvent content of 33.7%. Data from a

Selenomethionine ACPS-CoA crystal were also collected at three different wavelengths around the Se K edge (Table 1) on beamline X12-C of the National Synchrotron Light Source. Experimental phases were determined at 1.9Å and modified by solvent flattening. This also resulted in an experimental map that was of exceptionally high quality and using the $P2_1$ ACPS structure as a starting point, the protein was rebuilt according to the experimental density.

Structural Analysis of the Protein: The final model of ACPS (Figure 3) contains six copies of the protein arranged as two trimers. Each copy consists of residues 1-118 and the asymmetric unit also contains 471 solvent molecules, 8 sodium atoms and 19 chloride ions. The core of the protein is an α-helix (α4) composed of residues 43 to 64, which runs the entire length of the protein. One side of this helix is covered by an antiparallel β-sheet (A-Sheet) with topology β1, β5 and β4. A β-ribbon composed of strands β3 and β2, along with α3 combine to cover another side of helix α4. The encasement of α4 is completed by α1, α2 and a loop consisting of residues 66-75. This fold results in a majority of the surface of this protein being composed of β-strands. The superimposition of the Cα trace of each monomer is depicted in Figure 4 and shows that the six molecules of ACPS in the asymmetric unit have slightly different tracing of three loops (15-24, 80-88, 91-99) due to symmetry contacts. These molecules overlap with an average RMS delta of 0.497Å but since the beta sheets and helices are almost identical, the RMS delta improves to 0.168Å when the loops are omitted. There are no breaks in the electron density for any of the six monomers and only a few residues are not clearly defined. As a result of poor density beyond Cβ, the following residues were modeled as alanine: in monomer 1, LYS13, ARG70, and LYS107; in monomer 2, LYS13 and ARG70; in monomer 3, LYS13, GLU43, ARG70, and LYS107; in monomer 4, LYS13 and ARG70; and in monomer 6, LYS13 and ARG70. In monomers 2 and 4, the first glycine of the histidine tag, which was used as a

purification tool, is visible but no density is observed for the remainder of the his-tag. The electron density for Monomers 2, 3, 4 and 5 ends at residue 118. The electron density in Monomer 1 extends until residue 119 while it is traceable to residue 120 in monomer 6. The sequence of ACPS, SWISS-PROT accession number P96618, has a glutamine at position 96. The structure describe herein is of a mutant that has a proline instead of a glutamine at residue 96. This mutation is located in a flexible loop and is over 18Å from the CoA binding site. The mobility of this loop coupled with the fact that this protein retains its enzymatic activity suggests that this mutation is on a portion of the structure which is not associated with binding of ACP or CoA.

The crystal structure reveals that ACPS is a trimer. A predominant force behind the formation of the trimer appears to be burying of the 527 $Å^3$ surface of the A-Sheet. This sheet is composed of twenty seven amino acids and of the fifteen that are exposed, six are hydrophobic. The exposed ends of strand β1 are hydrophilic (TYR3 and GLU11) but the residues exposed on the interior are hydrophobic (ILE2, ILE5, LEU7 and ILE9). Likewise, the exposed ends of strand β5 are hydrophilic (TYR109 and GLU117) and in addition, the residue in the center of the strand, GLN113, is also a polar residue. The two remaining exposed residues of strand β5, ALA111 and VAL115, are hydrophobic. Unlike either β1 or β5, all the exposed residues of strand β4 are hydrophilic (HIS100, SER102, THR104, and THR106).

In this trimer, the A-sheet of each molecule comes together to form an arrangement similar to a three-faced beta barrel (Figure 4). This packing results in strand β1 from the first A-sheet packing against strands β4 and β5 of the second A-sheet. Strand β1 of the second sheet then packs against strands β4 and β5 of the third sheet while strand β1 of the third sheet packs against strands β4 and β5 of the first A-sheet. This results in the formation of an elongated three-faced β-sandwich. The packing at the junction between monomers forces the main chain

of strand β1 to point in the same direction as the polar side chains of strand β4. The interface where the β-sheets meet between two of the molecules of the trimer is therefore exceptionally polar. The area above this intersection is open to solvent and is at the bottom of a bowl-shaped depression in the surface of the protein assembly. This depression measures approximately 14Å in width, 20Å in length, and 8Å in depth and is where CoA binds to this protein. Examination of an electrostatic representation of this depression shows that the bottom of the cavity is anionic in nature while the sides are either cationic or hydrophilic. In this structure, the cavity is filled with numerous water molecules as well as three chloride ions and one sodium ion. The sodium ion is located at the intersection of two A-sheets and has as ligands residue HIS105 from one monomer and residue ILE9 from another monomer. There is a sodium ion in each of the six cavities present in the asymmetric unit of the $P2_1$ form and in each case, the ligand architecture is identical. Aside from the packing of the β-sheets, the only other interactions (both VDW and ionic) between the molecules of the trimer occur between residues of two loops containing residues 65-67 from one monomer and residues 84-86 from another monomer.

The final model of ACPS-CoA in the rhombohedral crystal contains residues 1-118, one molecule of coenzyme A, 99 solvent molecules, two calcium atoms, and one chloride ion. Similar to the ACPS structure alone, the electron density extends to residue 118 and there are no breaks in the density between residues 1 to 118. The side chains for residues LYS23, ARG70, ARG118 were not visible past Cβ and have been modeled as ALA. The binding of CoA has not resulted in any changes in the secondary structure of the protein since the same trimeric arrangement of the protein molecules described in the $P2_1$ space group above is also present in this structure. The monomer of ACPS in this structure of the ACPS-CoA complex is positioned such that the molecular three-fold axis is

coincident with a crystallographic three-fold axis. The trimer in this structure is generated by symmetry operators (-Y, X-Y, Z) and (-X+Y, -X, Z)

In this structure, the metal binding site described in the monoclinic structure is not present. Instead, there are two six-coordinate metal binding sites that are both occupied by calcium ions. The first calcium is found at one end of the 3-sided β-sandwich and sits on a special position (z=0). Only two of its ligands come from the molecule in the asymmetric unit of this structure, GLU108 and HOH21. Two symmetry-related copies of GLU108 and HOH21 finish out the coordination shell for this calcium. The site in which the sodium ion is found in the monoclinic ACPS structure is now occupied by the side chain of LYS62. As a consequence of this rearrangement, a second metal binding site is formed in the cavity just 5Å above that location. GLU58 and ASP8 occupy two equatorial sites and the α2 phosphate from the Coenzyme A pyrophosphate occupies an axial coordination site on the calcium. It is this metal binding site which is the location of the magnesium ion required for the enzymatic activity of this protein.

CoA binding site: The depression described in the monoclinic ACPS structure is the location of the active site of this protein. The rearrangement associated with moving LYS62 into the sodium-binding pocket allows two residues, ASP8 and GLU58, to define a binding pocket along the anionic ridge between the A-sheets into which a divalent cation fits. The calcium occupying this binding site serves to anchor the CoA in the pocket by coordinating to the α2 phosphate of the CoA pyrophosphate. The adenosine ring of CoA fits snugly into a pocket formed by a loop consisting of residues 80-88 and the C-terminal end of the α4 helix along with the loop immediately following (residues 62-70) of a symmetry related molecule. The 3'-phosphate of CoA is held in place by the protein through hydrogen bonds provided by the side chains of ARG53 and HIS105. The interactions between the pantetheinyl group and the protein are predominately van der Waals interactions.

The sulfhydryl group rests in a hydrophobic pocket formed by the aliphatic portion of the side chains of ARG28, MET18, PHE25, ILE29, PHE54 and PHE74. These interactions are detailed in Figure 5.

Superimposition of ACPS dimer and Sfp: The molecular coordinates for Sfp were obtained from the protein data bank, code 1QR0. All solvent molecules were ignored during the alignment calculations. The ACPS dimer and Sfp molecules were manually aligned using QUANTA (Molecular Simulations, Inc.) and the resulting overlapped coordinates written to disk. These coordinates were then read into 6D_LSQMAN and the alignment refined in an iterative process. When the alignment process converged, the ACPS dimer and Sfp superimposed with a RMS of 2.19Å.

When examining the coordinates of Sfp within QUANTA, it was evident that a loop corresponding to residues 83 to 94 had adopted an extended conformation in the C-terminal side of the molecule. The corresponding loop on the N-terminal side of Sfp clings to the protein body in an identical fashion to that found in ACPS. Therefore the superimposition was repeated with this C-terminal loop excised from both structures. The superimposition of the structures then improved to a RMS of 1.81Å. When the coordinate set used in the alignment was limited to those residues within 8Å of the CoA, the RMS remained at 1.81Å. However, when the coordinate set was limited to those atoms within 4Å of the CoA binding site, the RMS improved to 1.66Å.

Differences between Sfp and ACPS: Sfp is a monomer consisting of 228 residues. ACPS is a monomer consisting of 121 residues. The sequence identity between Sfp and an ACPS dimer is quite low, as only 55 of the 226 residues in Sfp have an identical counterpart in ACPS (24%). A further 56 residues of Sfp have a counterpart in the ACPS dimer which have similar properties.

Sfp contains two domains. Both of these domains are similar to an ACPS monomer. Where the third monomer would bind in the ACPS triad, Sfp contains a twenty residue C-terminal extension.

Three molecules of ACPS combine to form a trimer with three active sites. Sfp does not aggregate and the active site is contained within the monomer.

In ACPS, residues 83 to 94 comprise a loop that packs tightly against the main protein body. In Sfp, the corresponding loop on the N-terminal domain (residues 67-83) packs in the same fashion. However, the same loop on the C-terminal domain protrudes out from the protein body.

The adenine, ribose and pyrophosphate from the CoA moiety are similar in the active site of both proteins. However, the pantetheinyl group is modeled closer to the adenine in the Sfp structure while in the ACPS structure it adopts an extended conformation.

Differences between Sfp and ACPS active sites: As shown in Figure 5, extensive interactions exist between CoA and ACPS. When compared with Sfp, some residues in the active site are in essentially the same location while others have moved dramatically. Six of the nine residues that have significant interactions with this part of the CoA are identical between the two proteins (ASP8, GLU58, LYS62, HIS105, GLY65). The residues that are not identical are ASN84, ARG53, and PHE49.

Those residues that are almost identically placed include ASN84 and GLY65 (TYR73 and GLY158 in Sfp). These residues move by only 0.2Å. ASP8 (ASP107 in Sfp) moves by only 0.4Å, while LYS62 (LYS155 in Sfp) moves by 1.3Å. The positions of the other residues in the active site are dramatically different. For example, GLU58 (GLU151 in Sfp) moves 2.4Å. In Sfp, this residue is hydrogen bonded to the pyrophosphate group of CoA as well as to the metal ion. In ACPS, it is only bonded to the metal ion.

Proline 87 (PRO76 in Sfp) assists in defining the cavity into which the adenine group is wedged. This residue moves 1.9Å. PHE49 is replaced by THR44 in Sfp. In the ACPS structure, this residue is not significantly interacting with the CoA. In Sfp, THR44 is hydrogen bonded to the phosphate directly attached to the ribose. This residue has moved 4.2Å. ARG53 is replaced by ASP48 in Sfp. In ACPS, ARG53 is hydrogen bonded to the phosphate that is directly attached to the ribose. The positioning of this residue differs 4.5Å between the two structures.

Example 2

(i) Materials and Methods

Equilibrium analytical ultracentrifugation experiments were performed on a Beckman XLI/XLA Analytical Ultracentrifuge operating at three rotor speeds of 20K, 26K, and 30K rpm. Equilibrium was judged to be achieved when no deviations in a plot of the difference between successive scans taken 3 hrs apart was observed, usually within 20 hours. Scans were recorded at 10°C and 25°C, and signal was detected using absorbance optics (285 nM) and interference optics. Samples were then loaded into six-channel cells at 3 different protein concentrations under argon. The self-association of ACPS as a function of pH (pH 5.2, 6.4, and 7.5) and the effect of the substrates CoA and ACP upon the molecular mass at pH 6.4 were characterized by sedimentation equilibrium measurements. After the experiments, 15% non-reducing denaturing gels were run to determine if irreversible aggregation of ACPS occurred during the equilibrium experiments which were performed typically over 4 days. The partial specific volumes of ACPS and ACP were calculated based on the amino acid composition, and the density of the solvent was calculated from the chemical composition of the buffer using the computer program SEDNTERP.

Isothermal titration calorimetry (ITC) was used to monitor the heat of binding observed upon addition of 8 μL aliquots of 269 μM ACPS in 100 mM Bis-Tris buffer (pH 6.4), to 1.38 ml of 10 μM ACP in 100 mM Bis-Tris, pH 6.4.

Equations: The molecular weight of the protein in the presence and absence of ligand was obtained from sedimentation equilibrium experiments using the following equation:

$$a_r = a_{r0}\exp[(\omega^2 M/2RT)(1-\nu\rho)(r^2-r_0^2)] + E$$

where:

a_r = concentration at radius r
a_{r0} = absorbance at reference radius x0
ν = partial specific volume of the macromolecule (mL/g)
ρ = density of the solvent
ω = angular velocity of the rotor (radians/sec)
E = baseline offset
M = gram MW of the macromolecule
R = gas constant
T = temperature

(ii) Results

Analytical ultracentrifugation is a classical biochemical method used to characterize the solution behavior of solutes. The technique relies on the principal property of mass and the fundamentals of gravitation, and can be used to obtain information about the solution molar mass, association constants, and stoichiometries by performing sedimentation equilibrium experiments. The monomer molecular weight of 15 kDa was obtained using a sample of ACPS in a solution containing 6 M of the denaturant guanidine hydrochloride (Table 2). Results from the equilibrium experiments with 100 μM ACPS and 100 μM ACPS/200 μM CoA at 6.4 pH and 25 deg. are presented in Figures 6A and B. The concentration gradient of ACPS across the cell readily fit the equation for the presence of a single species as evidenced by the random distribution of the

residuals, with a molecular weight of 22.5 kDa and 39.7 kDa, respectively. These molecular weights corresponded to a dimeric ACPS in the absence of CoA which then increases to a trimeric species in the presence of CoA. At 100 μM ACPS pH 5.2 and 6.4, the molecular weights are 22K and 25.8K, respectively (Table 2). When assuming the presence of monomer or trimer, the fits of the curves yielded greater than 90% dimer at pH 5.2 and 6.4.

15% nonreducing denaturing gels indicate the association is not due to covalent crosslinking of the subunits but self association, as greater than 95% of ACPS is monomeric before and after the centrifugation experiments (Figure 7).

The observation of a trimeric ACPS in the presence of CoA is in good agreement with the X-ray derived crystal structure of the CoA complex and supports the use of the trimeric structure for future drug screening assays, but also indicates the solution association interactions of ACPS with the two substrates CoA and ACP is a complex mixture of multiple species which may impact enzyme assay design and development.

Table 2

Apparent Molecular Weights of ACPS

Sample	pH	Temperature (°C)	MW
100 μM ACPS	5.2	10	22,000 ± 600
100 μM ACP	6.4	10	9,160 ± 300
100 μM ACPS	6.4	25	25,800 ± 900
300 μM ACPS/ 6 μM GuHCl	5.2	25	15,000 ± 400
100 μM ACPS/ 200 μM CoA	6.4	25	39,700 ± 700
MW based on amino acid composition ACPS: 13.7 kDa ACP: 8.8 kDa			

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All publications mentioned herein above, whether to issued patents, pending applications, published articles, or otherwise, are hereby incorporated by reference in their entirety. While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of the disclosure that various changes in form and detail can be made without departing from the true scope of the invention in the appended claims.